pH and Conformation Modulate Copper-Mediated Site-Specific Degradation of the IgG1 Hinge Region

BY

Zephania Kwong Glover

Submitted to the graduate degree program in Pharmaceutical Chemistry and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Arts.

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Chairperson: Dr. Jennifer Laurence

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Dr. Sreedhara Alavattam

_____

Dr. Russell Middaugh

Date Defended: June 11, 2015

The Thesis Committee for Zephania Kwong Glover certifies that this is the approved version of the following thesis:

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ABSTRACT

Fragmentation in the hinge region of an IgG1 monoclonal antibody (mAb) can impact product stability, potentially causing changes in potency and efficacy. Metals ions, such as Cu^{2+}, can bind to the mAb and undergo hydrolysis and/ or oxidation, which can lead to cleavage of the molecule. To better understand the mechanism of Cu^{2+}-mediated mAb fragmentation, hinge region cleavage products and their rates of formation were studied as a function of pH with and without Cu^{2+}. More detailed analysis of the chemical changes was investigated using model linear and cyclic peptides (with the sequence of SCDKTHTC) derived from the upper hinge region of the mAb. Cu^{2+} mediated fragmentation was determined to be predominantly via a hydrolytic pathway in solution. The sites and products of hydrolytic cleavage are pH and strain dependent. In more acidic environments, rates of Cu^{2+} induced hinge fragmentation are significantly slower than at higher pH. Although the degradation reaction rates between the linear and cyclic peptides are not significantly different, the products of degradation vary. mAb fragmentation can be reduced by modifying His, which is a potential metal binding site and a known ligand in other metalloproteins. These results suggest that a charge may contribute to stabilization of a specific molecular structure involved in hydrolysis, leading to the possible formation of a copper binding pocket that causes increased susceptibility of the hinge region to degradation.

ACKOWLEDGEMENT

I would like to express my extreme gratitude to Sreedhara Alavattam and Jennifer Laurence for their guidance and mentorship to enable this work, Aditya Wakankar for helpful discussions on the cyclic peptide, John Wang and Danielle Lieske for their thoughtful review, and Mary Cromwell and Jamie Moore for continued departmental support. In addition, I want to thank Amy Shen from Cell Culture for the mAb2 H224S mutant and Clifford Quan and Jeffrey Tom from the Peptide Synthesis Lab for the preparation of the synthetic peptides at Genentech.

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INTRODUCTION

Monoclonal antibodies (mAbs) are important protein therapeutic molecules for the treatment of a wide range of life-threatening diseases, ranging from oncology (cetuximab, trastuzumab), and inflammatory (adalimumab, rituximab) diseases to rare "orphan" disease indications (eculizumab for paroxysmal nocturnal haemoglobinuria).[1] IgG1 mAbs contain heterogeneity in size and charge generated during cell culture, purification, and processing and can accrue a variety of degradation products over storage.[2] During product development, characterization and monitoring of molecular attributes are necessary to demonstrate manufacturing consistency and shelf life prediction to ensure a potent and safe drug product.[3]

mAb fragmentation, or cleavage of the peptide backbone typically by hydrolysis, is a degradation process that occurs in a liquid drug product formulation. In particular, the IgG1 mAb hinge region of the heavy chain is prone to cleavage due to limited structural constraints and high solvent accessibility.[4] This highly conserved hinge consists of three regions: upper, core, and lower.[5] Fragmentation has been shown under solution storage to be typically confined to the upper hinge sequence,[6, 7] i.e. SC_{220}DKTHTC (Eu numbering[8]), which is linked to the light and inter-heavy chains through disulfide bonds at Cys_{220} and Cys_{226}, respectively.

Treatment of a mAb with enzymes such as papain and trypsin cleaves within the hinge region between the His-Thr bond[9] and Lys-Thr or Lys-Asp,[10] respectively, with the latter reaction influenced by nearby Asp residues.[11] Non-enzymatic fragmentation, on the other hand, can be observed by direct hydrolysis,[6] β-elimination,[7] and free-radical catalysis of peptide bond cleavage in mAbs,[12] and metal-mediated oxidative cleavage in peptides.[13] Fragmentation kinetics by non-enzymatic methods has been shown to vary with pH, where pH 6 has the lowest rate of

cleavage and rates increase in both more acidic and basic conditions,[14] and storage temperature.[15] Importantly, amino acids in the hinge such as His can facilitate fragmentation.[16]

In general, the peptide bond is inherently resistant to hydrolysis with a half-life of up to 267 years at 37 °C and 350 years at 25 °C, as determined by constructing pH-rate profiles with Gly-Val and Gly-Gly peptides studies, respectively, in uncatalyzed reactions at pH 7.[17, 18] Metal ions can enhance the rate of peptide cleavage and catalyze reactions when structurally positioned in the proper conformation for specific stereochemistry to occur.[19-23] A binding pocket, or active site, mediated by specific atoms within the amide bond and side chain moieties of residues can facilitate high-affinity binding and metal-dependent chemical reactivity.[24]

Cu^{2+} is known to form strong complexes with organic molecules due to their relatively high electron affinity,[25] especially with His and amide nitrogens.[26] In effect, Cu^{2+} can bind and enhance the degradation of proteins such as mAbs and hydrolyze small peptides and BSA.[27] Despite potential degradation reactions, Cu^{2+} is intentionally added as a component during mAb manufacture in the cell culture process to help maintain cell viability and to improve mAb titers.[28] Although most large-scale purification processes are able to remove most metals, trace amounts of Cu^{2+} (e.g. 15 ppb) may be sufficient to enable site-specific, metal-mediated mAb degradation.[29] Unintentionally, Cu^{2+} is introduced as an impurity often found in buffer components such as sodium chloride.[29] Previous work showed evidence of Cu^{2+}-mediated hinge cleavage of the mAb Campath 1H (alemtuzumab) in slightly alkaline pH.[30] The rate of cleavage was reduced in slightly acidic conditions (pH < 6) and was accelerated by increasing concentrations of cupric ion and higher temperatures.[30] With the addition of EDTA, the reaction was completely inhibited, which confirmed the involvement of metal ions in the cleavage. Allen et al. studied several peptides with the hinge sequence DKTHT at various pH values. They

observed that the rate of the reaction was slow below pH 5 and increased gradually from pH 7 to 9.5. Hydrolysis primarily occurred at the Lys-Thr peptide bond, [31] which is consistent wih the trypsin cleavage site. Allen et al. deliberately eliminated cysteine residues from the peptide to avoid non-native reactivity with the free cysteine sulfhydryl groups in the presence of Cu^{2+}.

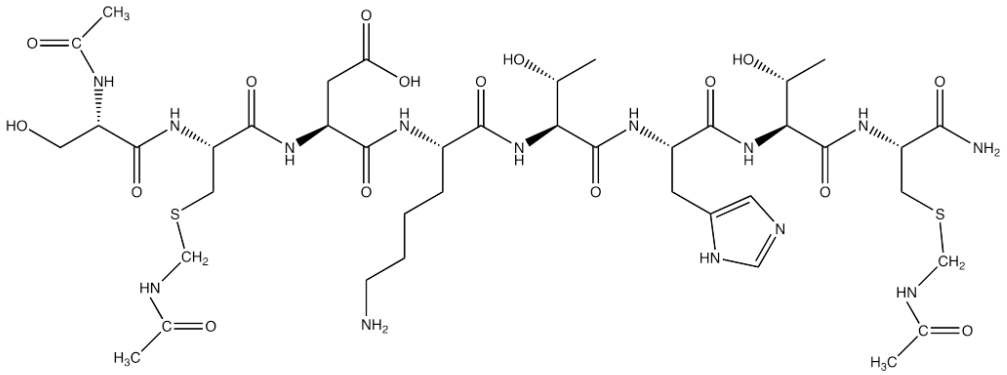
In our work, the dependence of a mAb to undergo fragmentation in the hinge region in the presence of Cu^{2+} was demonstrated and the mechanism of cleavage explored using model peptides derived from the hinge sequence. Hinge model peptides enabled the examination of site-specific interactions of hinge residues with Cu^{2+} without the complexities of a full-length mAb. The initial rates and pH dependence of degradation for the peptide were determined in the absence and presence of Cu^{2+}. A linear peptide (SCDKTHTC) with protected Cys that mimics the upper hinge region (Figure 1A) was used. In order to determine the effect of conformational rigidity on Cu^{2+}-mediated hinge fragmentation, degradation of a cyclized peptide (Figure 1B), in which the two Cys participate in an intramolecular disulfide linkage, also was examined. While a linear peptide closely resembles the primary sequence of the upper hinge, the embedded Cys in disulfide bonds can limit flexibility in the region. Evaluation of a structurally rigid model, such as a cyclic peptide, offers the opportunity to help illuminate the effect on degradation in solution by constraining the structure and/or imposing strain.

To investigate the importance of the hinge His_{224} for binding Cu^{2+}, a peptide variant and mAb mutant (H224S) was synthesized, and stability studies were conducted. Typically, mutational substitutions are made with small hydrophobic residues (e.g. Ala) to remove the effects of the side chain.[32] In our work, however, Ser was substituted for His to better understand the effect of hinge fragmentation mediated by another metal binding amino acid that possibly influences the Cu^{2+} coordination geometry.

Because Cu^{2+} is a redox active metal capable of oxidative reactions, Cu^{2+}-mediated fragmentation of the hinge peptide was considered with respect to both a hydrolytic and oxidative pathway. Based on the data presented herein, a Cu^{2+} binding site and cleavage mechanism is proposed.

A.

SC*DKTHTC* (linear)



B.

SCDKTHTC (cyclic)

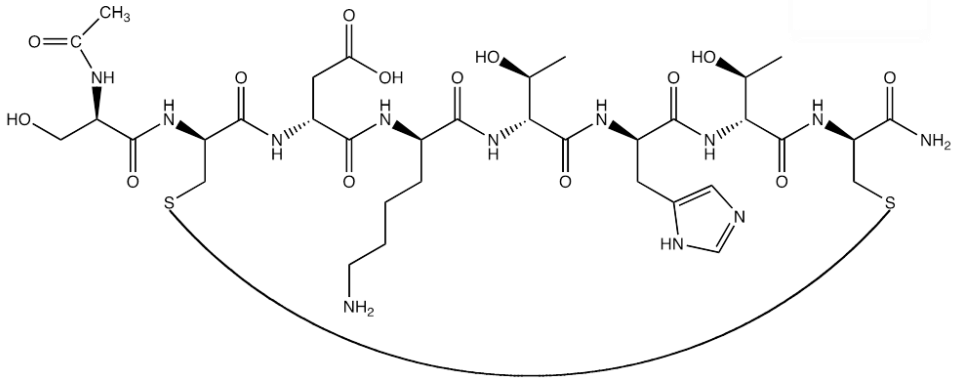


Figure 1. Model hinge peptides. A) Linear peptide with N-terminal acetylation, C-terminal amidation, and *Cys blocked with acetaminomethyl (ACM) groups. B) Cys deproptected and cyclized peptide.

EXPERIMENTAL SECTION

Materials

The recombinant humanized monoclonal IgG1 antibodies mAb 1, mAb 2, and mAb 2 (H224S) mutant were produced using Chinese hamster ovarian (CHO) cells and purified at Genentech (South San Francisco, CA). The amino acid substitutions were generated by site directed mutagenesis. The hinge region linear and cyclic octapeptide SC_{220}DKTHTC was synthesized at Genentech (South San Francisco, CA) with the N-terminus acetylated, C-terminus amidated, and the cysteines blocked with acetaminomethyl (ACM) to prevent dimer formation for only the linear peptide. The peptide was stored as a lyophilized powder at 5 °C and reconstituted for use with sterile water to make a 20 mg/mL stock solution. The reconstituted peptide was stored at -70 °C. The purity of the starting peptides was between 88- 98% as determined by RP-HPLC. All chemical reagents used were analytical grade or higher.

Sample Preparation

mAb 1 at 30 mg/mL (0.2 mM), mAb 2 wildtype (wt) at 1 mg/mL (0.007 mM), and mAb 2 (H226S) at 1 mg/mL were formulated in 20 mM sodium acetate or 20 mM histidine acetate at pH 5.3. mAb stocks were stored in liquid solutions at -70 °C. Test samples were incubated at 40 °C for up to 5 weeks with 0.04, 0.08, or 0.15 mM copper chloride ($CuCl_2$) (Sigma Aldrich, St. Louis, MO).

For all studies, the hinge peptide was diluted to 0.2 mg/mL (0.22mM) in either 10 mM sodium acetate, pH 5.0 (Sigma Aldrich, St. Louis, MO); 10 mM MES, pH 6.0 (Sigma Aldrich, St. Louis, MO); or 10 mM sodium HEPES, pH 7.0 (Sigma Aldrich, St. Louis, MO) buffer. The samples were stored for varying lengths of time (up to approximately 400 hours) at 50 and 60 °C with or without 0.15 mM $CuCl_2$.

For the oxidation studies, the hinge peptide was diluted to 0.2 mg/mL in 10 mM sodium acetate, pH 5.3. Samples were prepared with peptide in the presence of 0.15 mM $CuCl_2$ or peptide, 25 mM mannitol, and 0.15 mM $CuCl_2$, followed by storage at 60 °C for varying lengths of time (up to approximately 50 hours).

Due to the sensitivity, reproducibility, and precision of the assays (see methods section), purity of the starting material, controlled temperature monitored incubators (± 2 °C), and limited sample availability, the mAb and peptide Cu^{2+} incubation studies were perform n=1 for most cases, unless otherwise indicated. The R^2 values were determined for linear regression analysis of the rate data. Duplicate injections were performed to assess reproducibility of the assay for selected samples.

Size Exclusion Chromatography (SEC)

Size variant distributions of the mAbs were determined by size exclusion chromatography (SEC) using a TosoHaas Bioscience column G3000 SWXL, 7.8 x 300 mm, (South San Francisco, CA) with an autosampler set at 5 °C on an Agilent 1200 HPLC (Santa Clara, CA). All samples were injected at 50-µg load onto the column and eluted over 45 minutes with 0.2 M potassium phosphate, 0.25 M potassium chloride (pH 6.2) mobile phase at a flow rate of 0.3 mL/min. Protein elution was monitored at 280 nm. Results are reported as relative percent, which is the area of an individual peak divided by the total area under the curve. Data analysis performed on all chromatograms and integration was done using Thermo Scientific Chromeleon software (Sunnyvale, CA).

SEC was validated to evaluate the fragment observed in these studies. The repeatability and precision of the fragment was measured to be approximately ± 0.2% fragment assessed using

more than 30 injections, different mAb samples types, different analysts, and over several different days.

Reverse Phase High Performance Liquid Chromatography (RP- HPLC) and Mass Spectrometry (MS)

RP-HPLC was used to analyze the peptide on either an Agilent 1100 or Agilent 1200 HPLC (Santa Clara, CA) using a Vydac 218TP C-18 analytical column (4.6 x 250 mm) Grace Alltech, Columbia, MD) equilibrated at 82% and 18% of 0.1% TFA/ purified water and 0.1% TFA/ acetonitrile, respectively, then eluted with a gradient at 30 °C over 15 minutes at 1.0 mL/ min. The samples were injected onto the column at 2-µg load and monitored with UV absorption at 214 nm. Results are reported as relative percent peak area. Data analysis was done using Thermo Scientific Chromeleon software (Sunnyvale, CA). The samples were also injected onto the column at 10-µg load and analyzed by electrospray ionization MS using either an QStar Elite (AB Sciex, Framingham, MA) or a Orbitrap Elite™ Hybrid Ion Trap (Thermo Fisher Scientific, Waltham, MA) mass spectrometer. During the Orbitrap Elite mass spectrometer sample analysis, a splitter was used to lower the flow rate into the mass spectrometer to approximately 0.2 mL/min prior to coupling. Eluting products were ionized by positive mode electrospray ionization. MS/MS data was collected for the top 5 abundant peaks from every full MS scan. MS data analysis was performed using BioAnalyst (AB Sciex) or Xcalibur (Thermo Fisher) software. The identities of individual products were assigned by accurate mass measurement and confirmed by MS/MS data analysis.

RP-HPLC was able to detect changes in the peptide samples, with the repeatability and precision of the parent peptide measured to be up to ± 0.9% parent peptide assessed with 12

injections, different sample types, and over several days. Duplicate injections were performed at selected samples to confirm reproducibility of the assay (Table S1).

Bathocuproine Assay

The generation of Cu^{1+} from the reduction of Cu^{2+} in the samples was monitored at room temperature by measuring the absorbance at 483 nm in the presence of bathocuproine-disulfonic acid (BC, Nacalai Tesque), which specifically chelates Cu^{1+}. The reaction was carried out in 96-well plates containing 0.2 mg/mL peptide or buffer, 150 µM Cu^{2+}, 300 µM glycine, 20 mM sodium acetate at pH 5.3 and incubated at 60 °C (n=2). The standard curve was generated using 200 mM ascorbic acid and Cu^{2+} ranging from 0 to 100 µM. The sample mixture was incubated for 10 minutes at 37 °C in a 96-well plate and covered in foil prior to absorbance measurements on a plate reader Spectra Max.

Amplex Red Assay

Generation of hydrogen peroxide in the samples was measured using the Amplex Ultra Red Assay (Invitrogen, Carlsbad, CA) per the manufacturer's recommended procedure. The dye, horseradish peroxidase (HRP), reacts in a 1:1 ratio with H_2O_2, when added to the sample and results in the production of the fluorescent oxidation product resorufin. The reaction was carried out in a 96-well plate containing 0.2 mg/mL peptide, 150 µM Cu^{2+}, 300 µM glycine, 20 mM sodium acetate at pH 5.3 after incubation at 60 °C for 24 hours and quantified (n=3) using a Spectra Max M2Microplate Reader (Molecular Devices, Sunnyvale, CA) with excitation and emission set at 560 and 590 nm, respectively. Standard curve ranging from 0 to 20 µm H_2O_2 was generated to determine the final H_2O_2 concentrations.

RESULTS

mAb 1 Degradation in the Presence of Cu^{2+}

mAb 1 was treated with Cu^{2+} in sodium acetate or histidine acetate buffer at pH 5.3, stored at 40 °C for up to 5 weeks, and monitored by SEC to detect fragmentation in the hinge region. The SEC chromatographic profile of mAb 1 in sodium acetate buffer containing various Cu^{2+} concentrations is shown in Figure 2.

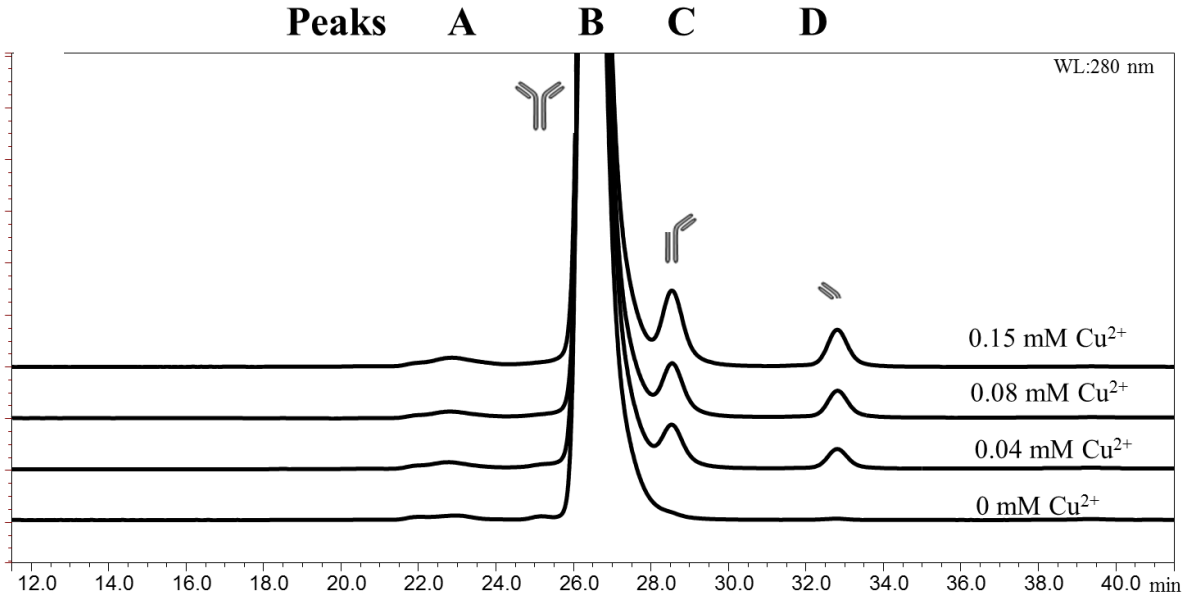


Figure 2. SEC chromatographic profile of IgG1 mAb 1 fragments in sodium acetate pH 5.3 in various copper concentrations after 1wk @ 40 °C as detected by absorbance at 280 nm A) High molecular weight species, B) monomer, C) fragment missing Fab arm (Fc + Fab), D) Fab arm.

The identity of these SEC peaks for mAb 1 were previously determined using mass spectrometry.[33] Addition of Cu^{2+} increased the rate of mAb 1 fragmentation as shown in Figure 3A, resulting in generation of the Fab arm and the single-arm antibody (Fab + Fc) from the parent mAb 1. Increased fragmentation with the addition of Cu^{2+} to mAb 1 in sodium acetate buffer was clearly observed and was not detected when formulated with histidine acetate buffer

(Figure 3B)

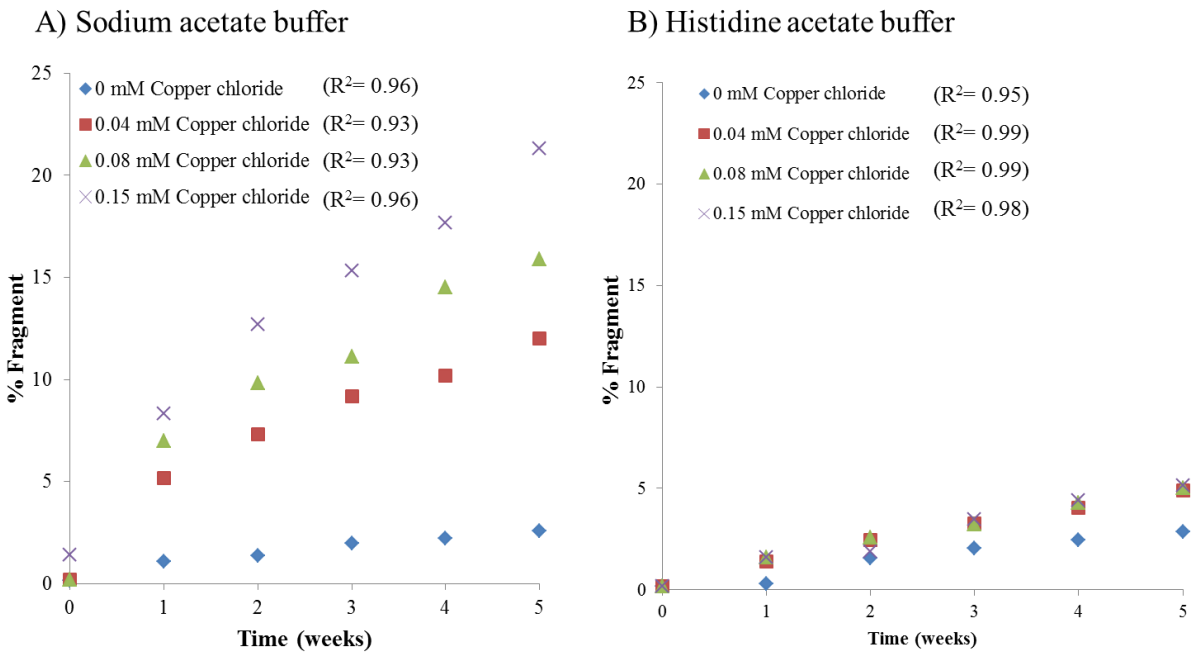


Figure 3. SEC analysis of total fragment formation for mAb ± Cu^{2+} during 40 ^{o}C storage at pH 5.3 in A) sodium acetate or B) histidine acetate.

The increase in total fragmentation (sum of peaks C and D) was tracked as a function of time to quantify the rate of degradation by linear regression (Table 1). In the presence of Cu^{2+}, mAb 1 in sodium acetate buffer exhibited significantly more fragmentation than in histidine acetate. In sodium acetate buffer, the increase in molar ratio of Cu^{2+}: mAb 1 to 1:9, 1:4, or 1:2 resulted in an approximate 5-, 6-, or 8-fold increase in fragmentation, respectively, as compared to the control (no Cu^{2+}, mAb 1 only). In contrast, mAb 1 in histidine buffer plus 0.038 or 0.15 mM Cu^{2+} showed an approximate 2 to 3-fold increase in fragmentation as compared to the control (Table 1).

Table 1. Linear regression fit of the initial fragmentation rates for mAb 1 ± Cu^{2+} at 40 °C up to 5 weeks determined using SEC. *Total fragments = Peaks C + D as shown in Figure 2.

mAb concentration (mM)	Cu^{2+} concentration (mM)	Fragmentation rate at pH 5.3 (% total fragment increase*/week)	
		Sodium acetate	Histidine acetate
0.33	0	0.46	0.58
	0.04	2.2	0.92
	0.08	2.9	0.94
	0.15	3.7	1.0

Peptide Degradation Rates in the Presence of Cu^{2+}

To better understand hinge fragmentation of the mAb in the presence of Cu^{2+}, a linear octapeptide derived from the upper hinge region, SC*DKTHTC* (Figure 1A; *acetaminomethyl (ACM) protecting group on Cys to block thiol reactivity) was synthesized and studied at pH 5, 6, and 7. Loss of the parent (intact) linear peptide incubated in the presence of Cu^{2+} at 50 °C was monitored using RP-HPLC to determine the initial rate of intact peptide loss by linear regression. The loss of the linear and cyclic intact peptide resulted in a heterogeneous mixture of degradation products. The linear peptide showed the lowest rate of peptide loss at pH 5 (Table 2). As the pH increased, the rate loss of the intact peptide also increased. The initial rates of intact peptide loss for 0.15 mM Cu^{2+} to 0.22 mM peptide at pH 5, 6, and 7 were approximately 3, 53, and 47-fold higher respectively than the corresponding control (no Cu^{2+}) reaction.

Table 2. Initial peptide degradation rates at 50 ^{o}C with and without Cu^{2+}

Initial degradation rate[a] (% intact peptide loss/ hour)			
	Linear		Cyclic
pH[b]	No Cu^{2+}	+ 0.15 mM Cu^{2+}	
5	0.053	0.20	0.081
6	0.043	2.3	--
7	0.060	2.8	1.9

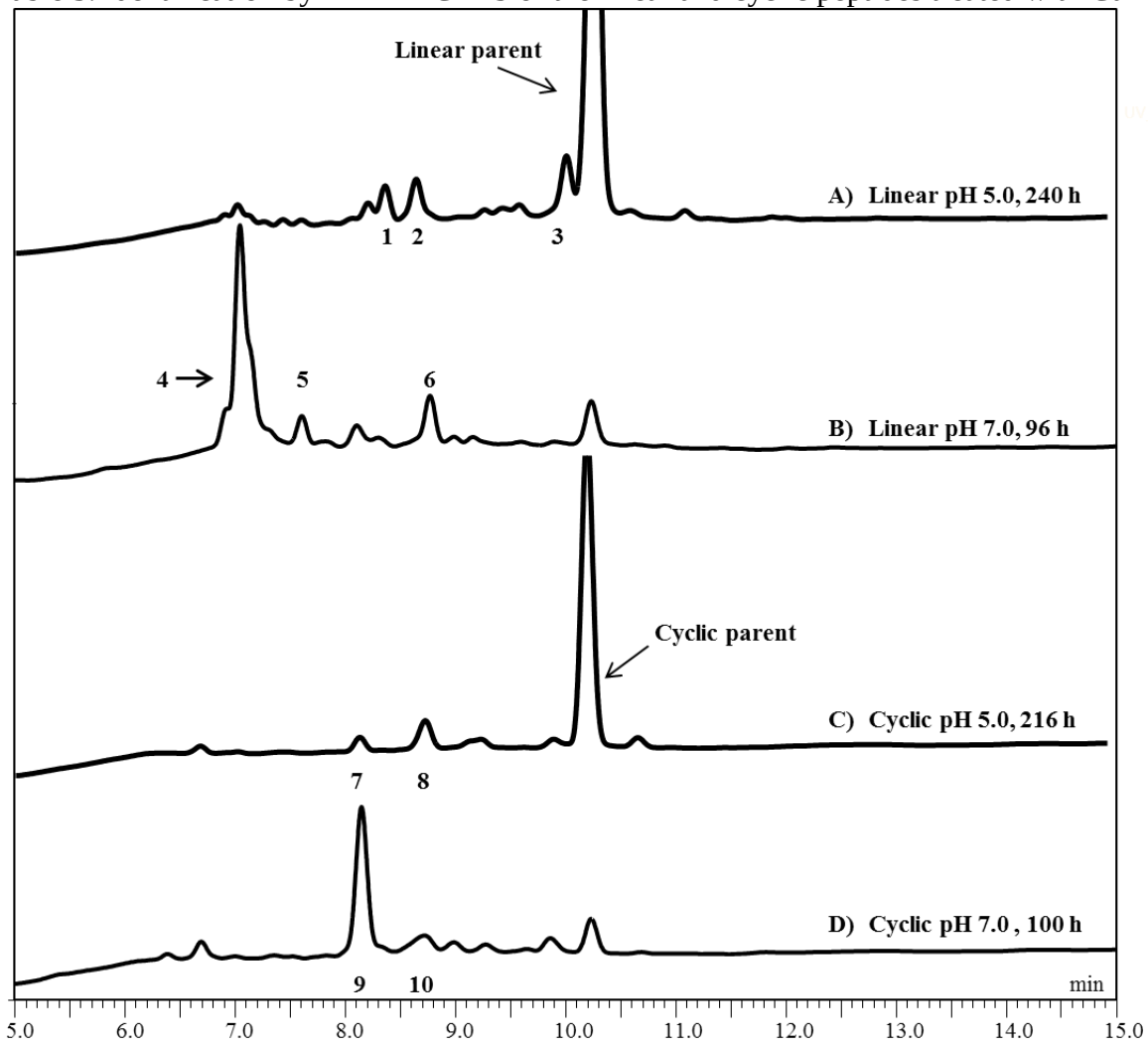
[a] Linear regression fit based on the intensity of the intact parent peptide peak obtained using RP-HPLC

[b] Incubated in sodium acetate (pH 5), MES (pH 6), or HEPES (pH 7) buffer

To identify the degradation products of the linear peptide in sodium acetate at pH 5, RP-HPLC and RP-HPLC- MS were performed. At pH 5, species with masses of 948-, 702-, and 1076-Da are detected beneath peak 1, 2, and 3, respectively (Table 3 and Figure 4). The 948-Da species suggests a +32 oxidation product of the intact peptide with the formation of a succinimide ring at the Asp residue, while the 702-Da species is the cleavage fragment DKTHTC, and the 1076-Da species is an Asp isomer of the intact peptide (Figure S2). A minor amount of dehydroalanine (MW = 900 Da) was also observed beneath peak 1 (MS data not shown). The +32 products of the intact peptide were observed when the Cys blocking groups were disassociated from the peptide and the Cys thiol groups get oxidized. Cys oxidation reaction commonly occurs in proteins as reported by Giles et al.[34] The decoupling reaction

between the blocking group and Cys are not well understood and will need further investigation. Detection of the DKTHTC peptide fragment (MW = 702 Da) confirms that cleavage occurs on the N-terminal side of Asp, suggesting a hydrolytic reaction is responsible for cleavage of the Cys-Asp bond. A more hydrophilic peak was observed at 10.0 minutes (peak 3), which corresponds to a 1076-Da species (Figure S2). This is the same mass as the intact peptide and is expected to be SC*isoDKTHTC*, which is a result of isomerization at the Asp residue. As expected, the change in the intensity of this peak was dependent on temperature and pH, but independent of the presence of Cu^{2+} (data not shown).

Table 3. Identification by RP-HPLC-MS of the linear and cyclic peptides treated with Cu^{2+}.



[a]Linear and cyclic peptide analysis performed on a Qstar and Orbitrap Elite, respectively.
[b] Observed values are after conversion to zero charge state.
[c]C-terminal amidated and N-terminal acetylated.
[d]Cys are ACM blocked (*).
[e]Cyclic peptides are disulfide linked.
[f]Asu= Succinimide
[g]pD= pyruvoyl

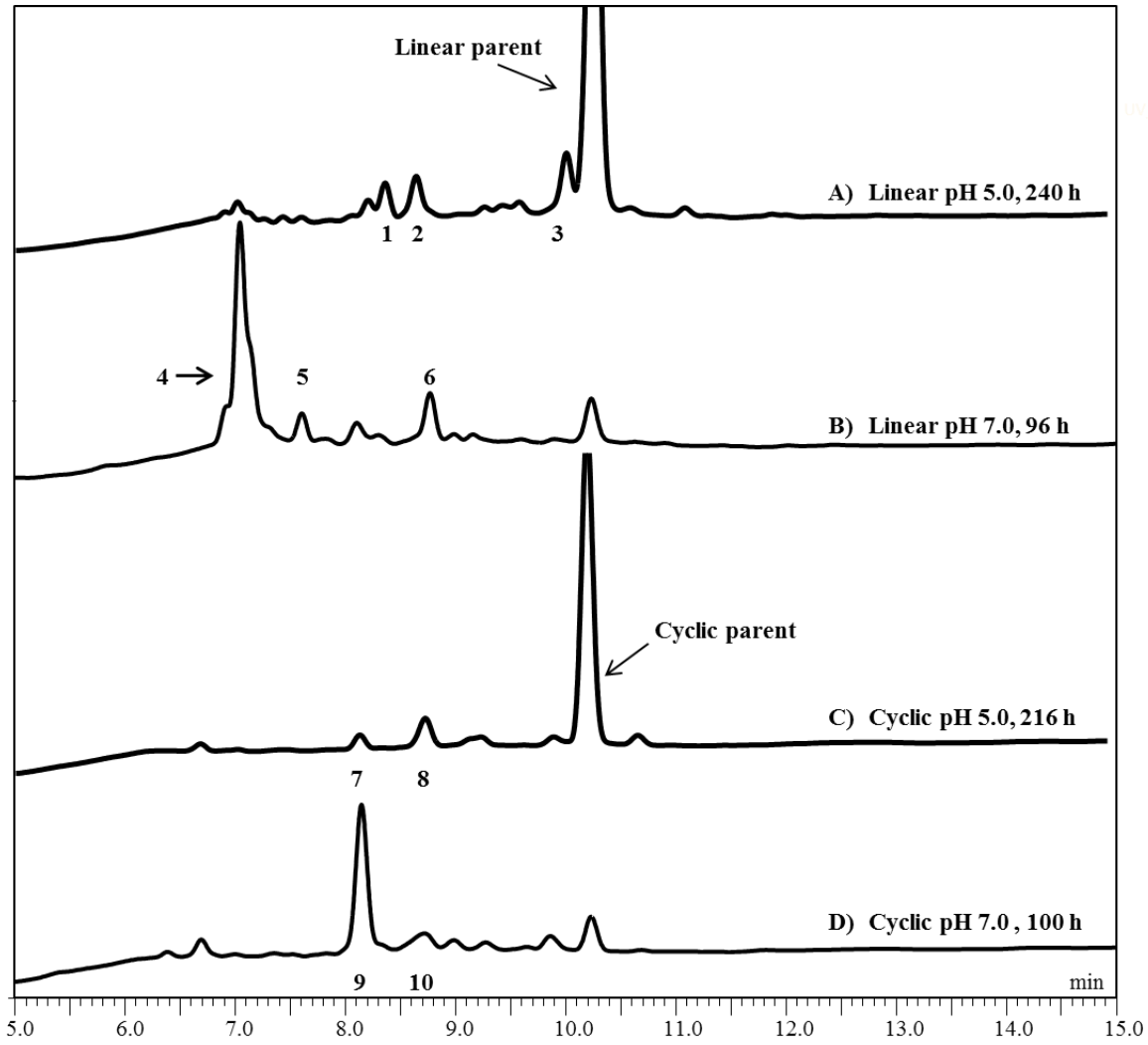


Figure 4. RP-HPLC chromatographic profiles of hinge peptides showing Cu^{2+}-induced degradation at 50 °C, as detected by absorbance at 214 nm, in either pH 5 sodium acetate buffer or pH 7 HEPES buffer. A) linear pH 5, 240 h, B) linear pH 7, 96 h, C) cyclic pH 5, 216 h, and D) cyclic pH 7, 100 h. Time point were selected to show adequate formation of degradation peaks because the rate and extent of degradation are pH specific. Similar chromatographic profiles are observed at pH 6 as compared to pH 7 for the linear peptide under similar conditions. The number of each labeled peak corresponds to that identified in Table 3.

Formation of the degradation products is Cu^{2+} dependent at pH 7 in HEPES buffer when incubated at 50 °C and the results are summarized in Table 3. At pH 7, the major degradation products were the THTC* (530 Da) and SC*DK (564 Da) fragments detected beneath peaks 4 and 5, respectively (Figure 4 and S3). The hydrolytic cleavage site between Lys and Thr was

consistent with previous Cu^{2+}-induced hinge region degradation studies at slightly alkaline pH.[30] Peak 6 contains a 773-Da species, which is most likely the C-terminal fragment DKTHTC* or DKTHTC modified with a pyruvoyl group (pDKTHTC, Figure S3). Furthermore, a small amount of a 900-Da species also eluted with peak 6, suggesting the formation of a dehydroalanine derivative (i.e. SdADKTHTC, data not shown). The observation of these species is consistent with a single chemistry; both species are intermediate products of beta elimination at either of the Cys residues.[7] Similar products were observed at pH 6 in MES (data not shown).

A deprotected, cyclized version of the hinge peptide, linked by an intramolecular disulfide bridge, was also prepared to assess the impact of molecular conformation, in the form of a more rigid and less flexible model, on the stability in similar conditions as the linear peptide (Figure 1B). In presence of 0.15 mM Cu^{2+}, initial rates for loss of the intact cyclic peptide are slightly lower compared to the linear peptide at pH 5 and 7 (Table 2). The degradation products of the linear and the cyclic peptides, however, are distinctly different and summarized in Table 3. At pH 5, two new peaks 7 and 8 were observed when the cyclic peptide was incubated in presence of 0.15 mM Cu^{2+} (Figure 4). In the absence of Cu^{2+} the rate of intact peptide loss was extremely low (data not shown). Peak 7 contains a +18 Da product of the intact peptide (950 Da), while the peak 8 contains a species with the same mass as the cyclic intact peptide (932 Da, Figure S4A). Similarly, at pH 7 two peaks were observed that correspond to a 950-Da (peak 9) and a 932-Da (peak 10) species (Figure 4, mass spectrum not shown). These peaks were determined by MS to be the same products as at pH 5, such that the peaks 7 and 8 were analogous to the peaks at 9 and 10 at pH 7. Interestingly, the Asp related N-terminal hydrolysis and isomerization products generated when the linear peptide was incubated in presence of Cu^{2+} under similar conditions were not observed with the cyclic peptide. The +18-Da species was

further analyzed using LC-MS/MS to confirm that Cu^{2+} induced a hydrolytic degradation between Lys and Thr at both pH 5 and pH 7, in contrast to what was observed in the reactions of Cu^{2+} with the linear peptide. To confirm the cleavage was indeed between Lys-Thr, a tryptic digest of the cyclic peptide was performed followed by LC-MS/MS. The results indicate the formation of the same product (950-Da species), which further validates the Cu^{2+} reaction leads to cleavage between the Lys-Thr (Figure S4B).

The Influence of the Histidine-224 on Hinge Region Stability

To investigate the importance of the His_{224} in the hinge for binding Cu^{2+}, a peptide variant (His to Ser) was synthesized and stability studies were conducted. Slower degradation of the main peak with the variant peptide compared to the wild-type peptide was observed (data not shown). To confirm whether these linear peptide results translate to the full-length antibody, an IgG1 mAb (mAb 2 wt) with the hinge region His mutated to Ser (mAb 2 H224S) was also studied for hinge fragmentation. mAb 2 wt and mAb 2 H224S were subjected to 40 °C storage up to 2 weeks with and without Cu^{2+} in sodium acetate buffer at pH 5.3. The relative rates of degradation for mAb 2 wt and mAb 2 H224S are shown in Figure 5. In the presence of Cu^{2+}, mAb 2 H224S showed a 3-fold decrease in the rate of fragmentation compared to mAb 2 wt. mAb 2 H224S with and without Cu^{2+} and mAb 2 wt without Cu^{2+} showed virtually no change up to 7 days. However an increase in fragmentation was noticed thereafter in these samples.

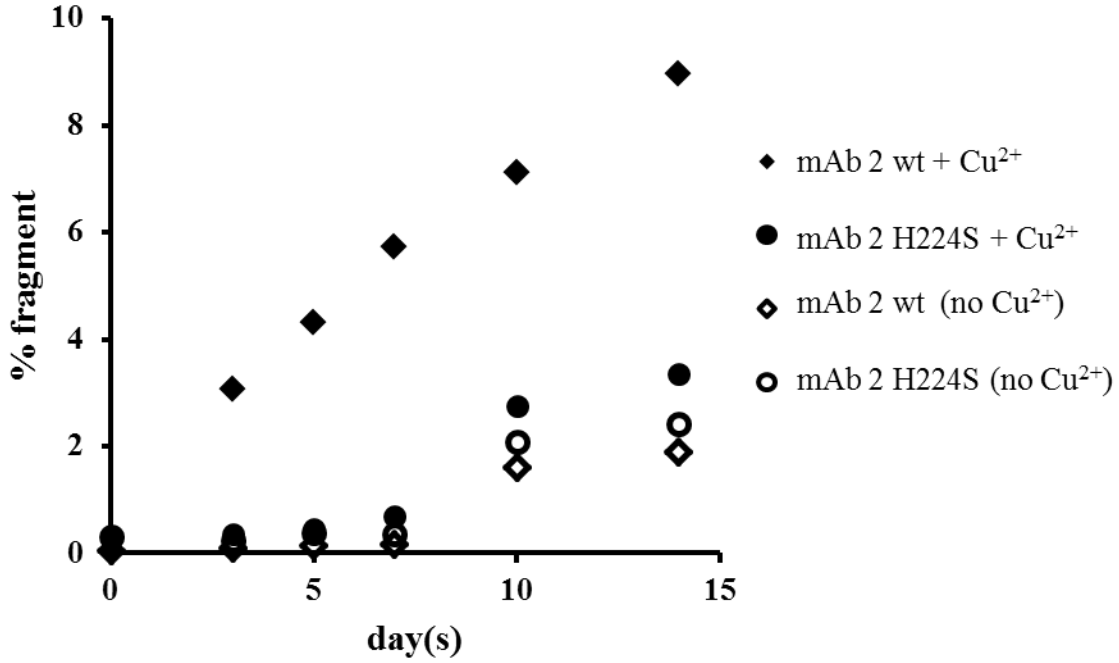


Figure 5. Plot of the total fragment data for mAb 2 and mAb 2 H224S, as determined by SEC. Samples were incubated in 20 mM sodium acetate, pH 5.3, in the absence or presence of 0.04 mM Cu^{2+} and stored at 40 °C. Aliquots were examined at various time points for up to 2 weeks. The R^2 values for the mAb 2 and mAb 2 H224S incubated with Cu^{2+} are 0.97 and 0.84 and without Cu^{2+} are 0.78 and 0.80, respectively.

The Impact of Reactive Oxidative Species on the Hinge Region

To test whether a free radical is responsible for cleavage of the hinge region, the Cu^{2+}-treated linear peptide was incubated with and without the hydroxyl radical scavenger mannitol in sodium acetate buffer at pH 5.3. The RP-HPLC results show a similar degradation pattern for both samples (Figure S5). To demonstrate that the reaction was not attributed to single electron transfer to Cu^{2+}, a bathrocuproine binding assay was also performed.[35] Incubation of the peptide in the presence 0.15 mM Cu^{2+} (n=2) after 24 hours at 60 °C resulted in 21 and 22 µM Cu^{1+} and at T0 resulted in 1 and 1 µM Cu^{1+}, indicating no appreciable amount of Cu^{2+} reduced to Cu^{1+}. Subsequently, the Amplex Red assay showed very little formation of H_2O_2 (2.9 ± 0.03 and 1.0 ±

0.04 µM H_2O_2 for the peptide at T0 and after 24 hours treated with 150 µM Cu^{2+}, respectively), indicating that the reduction of Cu^{2+} to Cu^{1+} and subsequent formation of H_2O_2 is not a predominant pathway during the degradation of the hinge peptide.

DISCUSSION

Allen et al have previously shown that the hinge peptide lacking a Cys residue undergoes Cu^{2+} mediated fragmentation at pH 7 and higher. We were interested in understanding the fragmentation patterns in the pH range of 5-7 because most of the mAbs in clinical development are formulated in this pH range. We also investigated the role of Cys and the role of strain in this fragmentation chemistry. Since Cu^{2+} is a redox active metal ion, we wanted to test the possibility of both hydrolytic and oxidative fragmentation chemistry. As the Cu^{2+}-mediated fragmentation was site specific, we propose a metal binding site within the hinge region of the IgG1 mAb.

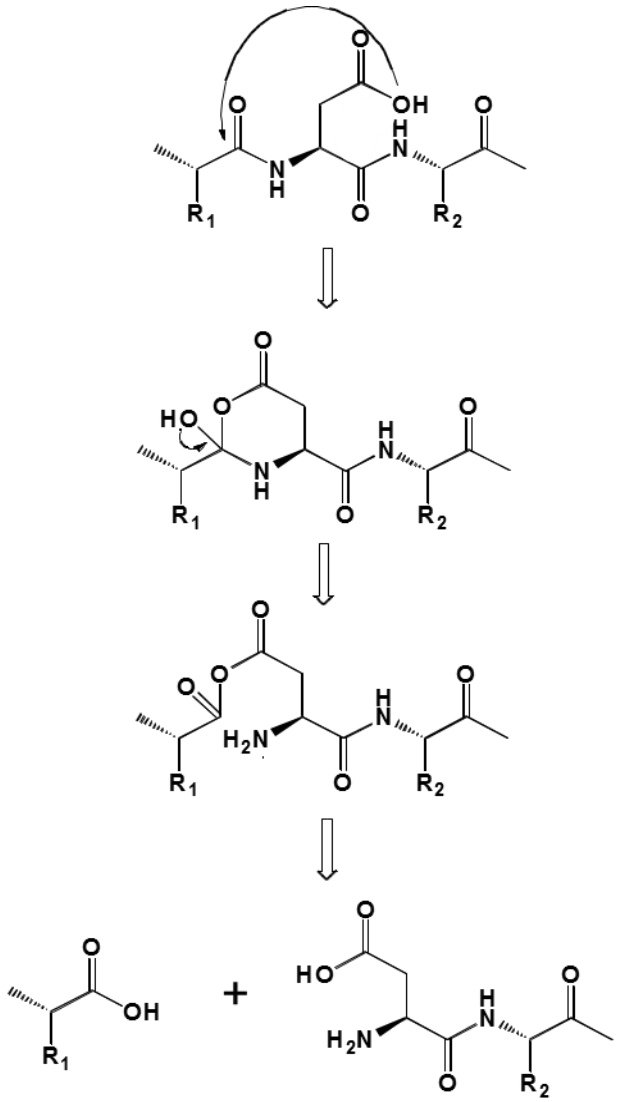
Fragmentation in the Linear Peptide

Due to the highly flexible and solvent-accessible nature of the upper hinge region[4, 33, 36] especially in an aqueous environment, an IgG1 mAb can be susceptible to non-enzymatic hydrolysis and degradation.[14] This reaction can be enhanced in the presence of metal ions such as Cu^{2+}, and buffers also may affect metal ion mediated fragmentation. As shown in Figure 3B, free L-His offered a protective effect against Cu^{2+}-mediated fragmentation. Inclusion of free His in the buffer has been shown to chelate Cu^{2+} and prevent the hinge cleavage reactions.[37] Salinas et al. further suggested preferential binding of the His to the mAb CH2 domain stabilizes the molecule against Cu^{2+} interactions to prevent fragmentation rather than direct chelation of Cu^{2+} by free His.[29] Interestingly, Ouellette et al. observed atypically high amounts of fragmentation of an IgG1 mAb containing a lambda light chain in histidine buffer at pH 6 incubated with Fe^{2+}. Subsequently Ouellette et al. showed that a mAb containing kappa light chain when treated with Fe^{2+} in a similar buffer showed no increase in fragments. These results were consistent with our observations that histidine buffer offered protective effects on a kappa light chain molecule in the

presence of Cu^{2+} (Figure 2 and Table 1).[38] The presence of an extra residue on the C-terminus of the light chain could influence the chemistry of the nearby hinge region and hydrolysis of the heavy chain, which could explain the difference in reactivity between a lambda and kappa light chain molecules in histidine buffer in the presence of redox active metals. The C-terminal region between a lambda and a kappa light chain differs such that a lambda light chain contains an additional Ser residue that the kappa light chain lacks. C-terminal Ser-containing peptides have been shown to hydrolyze rapidly via N to O acyl rearrangement in the presence of metals[19] and may be implicated for the marked increase in fragments. The results from our study using a mAb with kappa light chain indicated the chemical environment as controlled by buffer species has a significant impact on mAb cleavage.

The initial rates of degradation of the hinge peptides were pH dependent as shown in Table 2. At pH 5, a 702-Da species was observed upon incubating the linear peptide with Cu^{2+} in the acetate buffer. This species corresponds to a DKTHTC fragment that likely is formed due to a direct hydrolysis of the Cys-Asp bond. Cleavage at the Asp residue in peptides is known to be more susceptible to hydrolysis than other amino acids, especially under mildly acidic conditions.[39] Direct hydrolysis at the N-terminal side of Asp likely proceeds by a nucleophilic attack on the carbonyl of the n-1 amino acid residue (i.e. Cys), resulting in a 6-membered anhydride ring intermediate followed by hydrolytic cleavage of the bond between Cys-Asp, as shown in Scheme 1. Hydrolysis at the N-terminal side of Asp would then form the DKTHTC fragment. Since no pyruvoyl was detected at pH 5, the degradation pathway that formed the DKTHTC fragment most likely did not proceed via beta elimination through formation of dehydroalanine followed by further hydrolysis to form a pyruvoyl group. The reaction of Cys to dehydroalanine is typically driven at higher pH, such as pH 8.[7] However, trace amounts of a

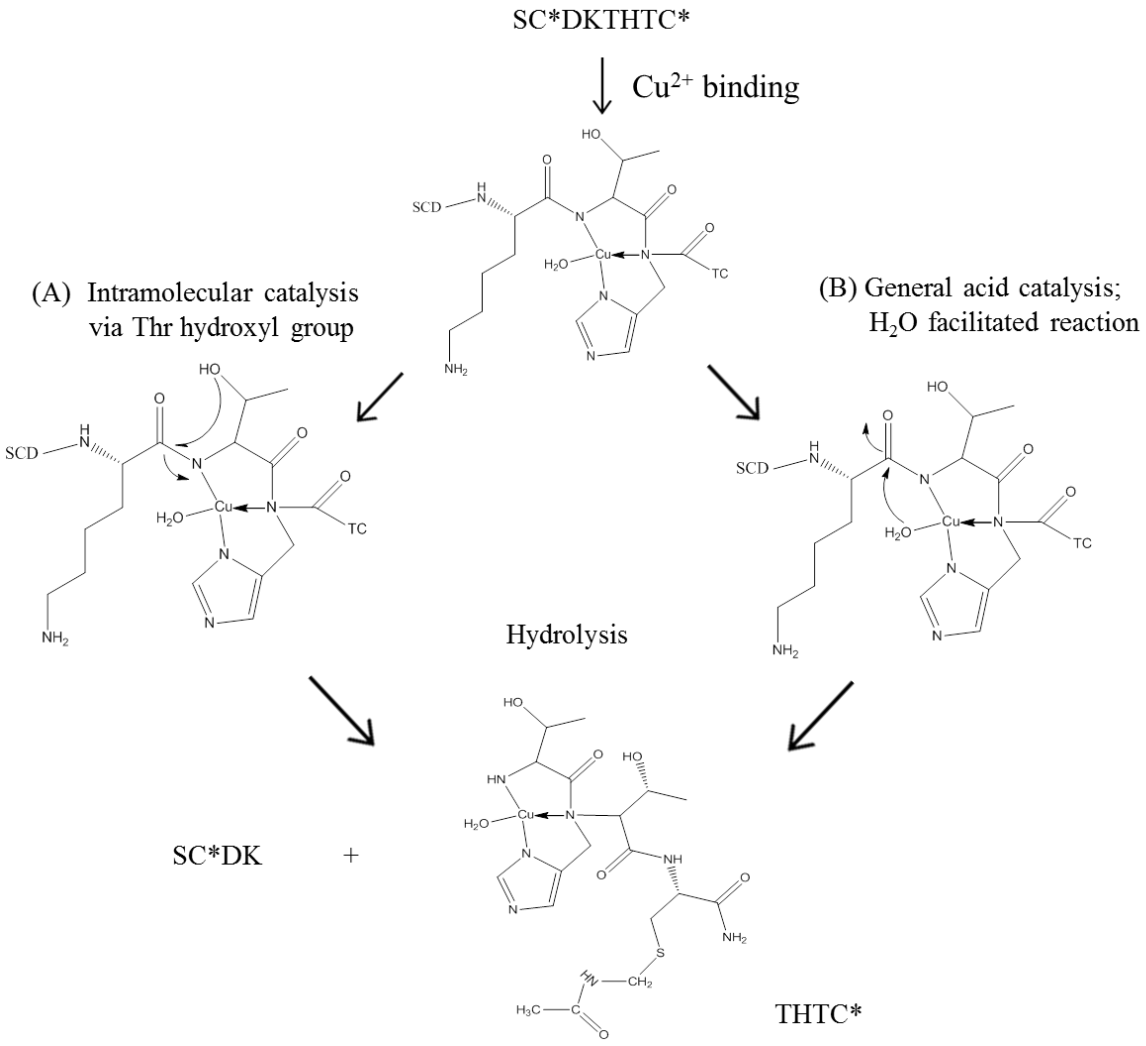
dehydroalanine species were observed even at pH 6 in our studies, indicating the presence of Cu^{2+} has an influence on the formation of this degradation product even at lower pH. The generation of dehydroalanine and pyruvoyl groups in the presence of Cu^{2+} at pH greater than 6 indicated that the Cys residues play an important role in the degradation of the linear peptide.



Scheme 1. Proposed mechanisms for fragmentation of the linear peptide at pH 5 showing direct hydrolysis facilitated by the Asp side chain.

In the presence of Cu^{2+}, Asp-mediated fragmentation in the linear peptide was prominent at pH 5 and to a lesser extent at pH 6 and 7, as indicated by the MS results (Figures S2 and S3). In addition to hydrolysis, Asp can undergo isomerization to iso-Asp via the formation of a succinimide ring intermediate, the rate-limiting step, and will degrade rapidly via hydrolysis at pH greater than 5.[40] At more acidic pHs, the Asp side chain (-COOH, pKa= 3.7) is more likely to be protonated than to persist as the carboxylate anion ($-COO^-$), which is less prone to isomerization. In the presence of Cu^{2+}, iso-Asp formation is still observed at pH 5. However, due to further degradation of the linear peptide at pH 5 in the presence of Cu^{2+}, it was difficult to accurately quantify the isomer peak. At pH 6 and 7, no isomer peak was observed in the presence of Cu^{2+}, and instead degradation of the linear peptide yielded SCDK and a substantial THTC fragment (peak 4). The cleavage sites were consistent with previous studies and confirmed by MS results in an IgG1[30, 37] and other peptides.[31] This cleavage could occur via two different pathways as shown in Scheme 2. Cu^{2+} may coordinate to the peptide via the histidyl nitrogen and two peptide amide nitrogens to facilitate cleavage on the N-terminal side of Thr in an intramolecular hydroxyl catalyzed reaction as shown in Pathway A. Here, an O-acyl intermediate would be formed between the Thr and the n-1 carbonyl carbon followed by hydrolysis of the ester. Alternatively, in Pathway B, general acid catalysis by the Cu^{2+}-bound water can occur without the involvement of the Thr hydroxyl group. Mutant studies by Allen et al. showed with the parent sequence FDKTHY that substitution of the Thr with Ala or Gly lowered the rate of cleavage compared to the parent, suggesting that the Thr-mediated intramolecular hydroxyl-catalyzed pathway was more likely than a general acid-catalyzed reaction to be responsible for hinge hydrolysis.[31] Thr or Ser can polarize the peptide bond via coordination and intramolecular attack of the hydroxyl group on the carbonyl carbon to form an ester intermediate in this N to O

acyl rearrangement reaction.[19] Studies with Cu^{2+} in myoglobin showed highly selective cleavage in the sequence QSHAT, where only the peptide bonds on the N-terminal side of the Ser and Thr residues were hydrolyzed.[41] Overall from our linear peptide studies, it is clear that at the slightly acidic pH (i.e. pH 5), the major cleavage site was the Cys-Asp bond (Scheme 1), whereas at pH 6 and 7, the Lys-Thr bond is cleaved (Scheme 2).



Scheme 2. Proposed mechanism for copper-induced fragmentation of the linear peptide at pH 6 and 7.

His coordinates Cu^{2+} readily in solution and in proteins such as serum albumin,[42] prions,[43] and superoxide dismutase (SOD)[44] to support high-affinity binding to Cu^{2+} through its imidazole nitrogens. In serum albumin, Cu^{2+} complexes with the histidyl nitrogen atoms in the imidazole ring and anchors the ion in a square planar arrangement together with its terminal amino group and the two successive backbone amide nitrogens in the peptide sequence.[45] Other amino acids containing hydroxyl groups (e.g. Ser, Thr) or carboxylic acids (e.g. Asp) can bind metal ions as well, though their geometry will be substantially different from that of His. Substitution of the His, if involved in Cu^{2+} binding, with the polar neutral amino acid Ser would impair binding to Cu^{2+} or result in a different configuration, ultimately leading to a reduced rate of fragmentation. This outcome was observed with the linear peptide in which His was substituted with either Ala or Ser, resulting in a significantly reduced rate of degradation (data not shown). These data clearly indicated that pH modulates the Cu^{2+}-mediated fragmentation pattern for the linear peptide and that Cys, Asp, and His play an important role in the degradation reaction.

Fragmentation in the Cyclic Peptide

Previously, Bogdanowich-Knipp et al. demonstrated the impact of peptide cyclization and its effect in solution stability.[46] The authors conducted studies using a cyclic RGD peptide at 50 °C that showed a marked increase in solution stability over the range of pH 3-7 as compared to the linear counterpart, with up to a 30-fold improvement at pH 7. The lack of flexibility in the cyclized peptide prevents Asp-mediated fragmentation via the carboxylic acid side chain to cleave the peptide backbone. The impact of cyclization of the hinge peptide on Cu^{2+}-mediated fragmentation was evaluated herein. The rigidity imposed by the cyclization of the peptide appears to affect the mechanism of fragmentation in the presence of Cu^{2+} rather than having a

significant impact on the rate of the same reaction as shown in Table 2. In the cyclic peptide, a single product with mass of +18 Da was observed at pH 5 and 7 in the presence of Cu^{2+} (Figures 4 and 5, respectively). This differs from the linear peptide where pH altered the degradation products and suggests that in the context of the full-length mAb conformation rather than the pH in this range has a larger influence on the cleavage reaction. The +18-Da products were characterized as a hydrolysis reaction between Lys-Thr in the cyclic peptide and confirmed using trypsin digest (Figure S4B). Rustandi et al. conducted mass spectrometry studies to show that the major cleavage site upon Cu^{2+} treatment is the Lys-Thr peptide bond within an IgG1 mAb in PBS.[37] Cyclization of the peptide increased the structural rigidity and interestingly prevented the attack by the Asp residue to mediate peptide backbone cleavage. As such, the susceptibility of the Lys-Thr site becomes dominant. After the Cu^{2+}-mediated fragmentation of the cyclic peptide results in SCDK and THTC fragments, and the resultant release of conformational strain, a succinimide ring product (-18 Da) was observed at both pH 5 and pH 7. Such a succinimide ring was not observed when control experiments without Cu^{2+} were carried out for extended periods of time in similar buffer systems (data not shown). Data generated using the cyclic peptide indicates that conformation plays a significant role in the Cu^{2+}-mediated fragmentation.

Reactive Oxygen Species dependent Cu^{2+} fragmentation is not a major pathway

Cu^{2+} is a redox active metal ion that has been implicated in both hydrolytic as well as oxidative cleavage of peptide bonds. The one-electron reduction of Cu^{2+} to Cu^{1+} in the presence of a reducing agent and molecular oxygen can lead to the generation of various reactive oxygen species (ROS; equations 1-4).[47] The generation of Cu^{1+} can be detected using the bathocuproine assay, while the generation of H_2O_2 can be detected using the Amplex assay.

$$Cu^{2+} + e^- \rightarrow Cu^{1+} \qquad \text{(Eqn 1)}$$

$$Cu^{1+} + O_2 \rightarrow Cu^{2+}(O_2^{-*}) \quad \text{(Eqn 2)}$$

$$Cu^{2+}(O_2^{-*}) + Cu^{1+} \rightarrow Cu^{2+}(O_2^{2-})Cu^{2+} \quad \text{(Eqn 3)}$$

$$Cu^{2+}(O_2^{2-})Cu^{2+} + 2H^{+} \rightarrow Cu^{2+} + H_2O_2 \quad \text{(Eqn 4)}$$

Additionally, Cu^{2+} in presence of hydrogen peroxide can generate hydroxyl radicals that can cause additional damage to the peptide backbone. Hydroxyl radicals generated in the presence of Cu^{2+} sulfate have been implicated in the cleavage of the IgG1 hinge region.[12] Our data using mannitol showed that there was no impact on fragmentation in the presence of this hydroxyl radical scavenger. Nonetheless, it has been shown that a hydroxyl radical generated in close proximity to the site of oxidation may be consumed before it can diffuse, which would render mannitol unable to quench the reaction.[48] Data from the Amplex red assay indicates that very little to no hydrogen peroxide was generated when the peptide was incubated with Cu^{2+}. The data from the bathocuproine assay showed only approximately 15% reduction of Cu^{2+} to Cu^{1+}, adding further support that single electron transfer and further generation of various ROS is not the major pathway in Cu^{2+}-mediated degradation of the hinge peptide. Taken together these data indicate the mechanism of hinge peptide fragmentation by Cu^{2+} is predominantly hydrolytic in nature.

Amino Terminal Cu^{2+} and Ni^{2+} (ATCUN)-like Motif that Can Facilitate Cu^{2+} Binding and Enhances Fragmentation in the Upper Hinge Sequence of an IgG1

The variant and mutation analyses emphasize the high degree of specificity that Cu^{2+} has for His in the hinge region. To achieve site-specific cleavage, a binding pocket that presents additional specific ligands to accomplish metal coordination in an appropriate geometry for Cu^{2+} binding would be expected. Crystal structures of Cu^{2+} peptides, such as the Cu^{2+}-GGH-methyl-amide complex,[24] confirm Cu^{2+} complexes often adopt square planar geometry. The GGH or ATCUN motif utilizes the amino-terminal nitrogen, the following two peptide backbone nitrogens, and the imidazole nitrogen of the His with particularly high binding affinity (i.e. Kd = 2.07×10^{-17}).[24, 49] While four nitrogen atoms are involved in metal ion binding in a square planar configuration, only 1.9% of His screened in as many as 1949 polypeptide chains were found capable of forming ATCUN like motifs,[49] substantiating that Cu^{2+} binding to IgG1 may not be a random event. IgG1 mAbs do not contain an ATCUN motif, but examination of the crystal structure of an IgG1 (PDB: 1HZH)[50] revealed that the hinge region sequence containing the peptide sequence studied herein adopts the appropriate geometry and distances to accommodate binding Cu^{2+} in a square planar organization. In Figure 6, the structure of the hinge region is shown and a Cu^{2+} ion is positioned in it in square planar geometry to illustrate the proposed Cu^{2+}-binding pocket. Combined with the optimal pH, buffer, and storage conditions, specific coordination of Cu^{2+} in this binding pocket would significantly enhance the rate of hydrolysis of the hinge region.

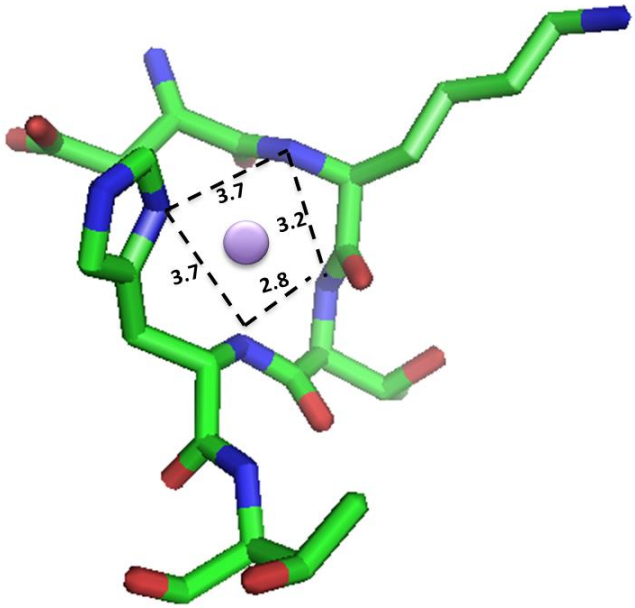


Figure 6. Model of Cu^{2+} bound to the DKTH sequence of the hinge region in IgG1. Square planar geometry of the Cu^{2+} ion (purple circle) is expected based on other copper-binding peptides and the crystal structure of IgG1 (PDB: 1HZH)[50], which shows these residues are poised to accommodate square planar coordination via the backbone amide nitrogens from Lys, Thr and His and the imidazole N from the His. Image created using PyMOL.[51]

CONCLUSIONS

mAbs in a liquid formulation are susceptible to binding and hydrolytic cleavage mediated by redox active transition metals such as Cu^{2+}. Accelerated degradation of the mAb mediated by Cu^{2+} was predominantly in the hinge region. Based on the published crystal structure of a full length IgG1 motif (1HZH),[50] the linear SCDKTHTC sequence has the potential to form a square planar coordination structure that potentially facilitates Cu^{2+} binding. Substitution of the His to other amino acids that do not contain an imidazole moiety but still bind metal ions prevents degradation. Collectively, these studies show that the amount of degradation products can be limited by a mAb formulation such as in histidine buffer at pH 5 or by mutating out key moieties

located within in the proposed metal binding site of the mAb. Such mutations may lead to a more stable molecule that can resist the detrimental effects caused by trace amounts of metals that are unavoidable during the mAb manufacturing process. On-going studies will include examination of the effects of other site-specific mutations on the hinge, the effects of various amino acid side chain residues, the role of redox inactive metal ions (e.g., Zn^{2+}) on mAb stability and whether the proposed Cu^{2+} binding site in the hinge region binds other metal ions as well.

REFERENCES

1. Beck A, Wurch T, Bailly C, Corvaia N. Strategies and challenges for the next generation of therapeutic antibodies. Nat Rev Immunol 2010; 10:345-52.
2. Liu H, Gaza-Bulseco G, Chumsae C, Newby-Kew A. Characterization of lower molecular weight artifact bands of recombinant monoclonal IgG1 antibodies on non-reducing SDS-PAGE. Biotechnol Lett 2007; 29:1611-22.
3. Martin-Moe S, Lim FJ, Wong RL, Sreedhara A, Sundaram J, Sane SU. A new roadmap for biopharmaceutical drug product development: Integrating development, validation, and quality by design. J Pharm Sci 2011; 100:3031-43.
4. Vlasak J, Ionescu R. Fragmentation of monoclonal antibodies. mAbs 2011; 3:253-63.
5. Padlan EA. Anatomy of the antibody molecule. Mol Immunol 1994; 31:169-217.
6. Cordoba AJ, Shyong BJ, Breen D, Harris RJ. Non-enzymatic hinge region fragmentation of antibodies in solution. J Chromatogr B: Anal Technol Biomed Life Sci 2005; 818:115-21.
7. Cohen SL, Price C, Vlasak J. Beta-elimination and peptide bond hydrolysis: two distinct mechanisms of human IgG1 hinge fragmentation upon storage. J Am Chem Soc 2007; 129:6976-7.
8. Edelman GM, Cunningham BA, Gall WE, Gottlieb PD, Rutishauser U, Waxdal MJ. The covalent structure of an entire gammaG immunoglobulin molecule. Proc Natl Acad Sci U S A 1969; 63:78-85.
9. Wang AC, Wang IY. Cleavage sites of human IgG1 immunoglobulin by papain. Immunochemistry 1977; 14:197-200.
10. Fan X, Brezski RJ, Fa M, Deng H, Oberholtzer A, Gonzalez A, Dubinsky WP, Strohl WR, Jordan RE, Zhang N, et al. A single proteolytic cleavage within the lower hinge of trastuzumab reduces immune effector function and in vivo efficacy. Breast Cancer Res 2012; 14:R116.
11. Siepen JA, Keevil EJ, Knight D, Hubbard SJ. Prediction of missed cleavage sites in tryptic peptides aids protein identification in proteomics. J Proteome Res 2007; 6:399-408.
12. Yan B, Boyd D. Breaking the light and heavy chain linkage of human immunoglobulin G1 (IgG1) by radical reactions. J Biol Chem 2011; 286:24674-84.
13. Kowalik-Jankowska T, Ruta M, Wisniewska K, Lankiewicz L, Dyba M. Products of Cu(II)-catalyzed oxidation in the presence of hydrogen peroxide of the 1-10, 1-16 fragments of human and mouse beta-amyloid peptide. J Inorg Biochem 2004; 98:940-50.
14. Gaza-Bulseco G, Liu H. Fragmentation of a recombinant monoclonal antibody at various pH. Pharm Res 2008; 25:1881-90.
15. Xiang T, Lundell E, Sun Z, Liu H. Structural effect of a recombinant monoclonal antibody on hinge region peptide bond hydrolysis. J Chromatogr B: Anal Technol Biomed Life Sci 2007; 858:254-62.
16. Yates Z, Gunasekaran K, Zhou H, Hu Z, Liu Z, Ketchem RR, Yan B. Histidine residue mediates radical-induced hinge cleavage of human IgG1. J Biol Chem 2010; 285:18662-71.
17. Smith RM, Hansen DE. The pH-rate profile for the hydrolysis of a peptide bond. J Am Chem Soc 1998; 120:8910-3.
18. Radzicka A, Wolfenden R. Rates of uncatalyzed peptide bond hydrolysis in neutral solution and the transition state affinities of proteases. J Am Chem Soc 1996; 118:6105-9.

19. Yashiro M, Sonobe Y, Yamamura A, Takarada T, Komiyama M, Fujii Y. Metal-ion-assisted hydrolysis of dipeptides involving a serine residue in a neutral aqueous solution. Org Biomol Chem 2003; 1:629-32.
20. Belczyk-Ciesielska A, Zawisza IA, Mital M, Bonna A, Bal W. Sequence-Specific Cu(II)-Dependent Peptide Bond Hydrolysis: Similarities and Differences with the Ni(II)-Dependent Reaction. Inorg Chem 2014; 53:4639-46.
21. Krezel A, Mylonas M, Kopera E, Bal W. Sequence-specific Ni(II)-dependent peptide bond hydrolysis in a peptide containing threonine and histidine residues. Acta Biochim Pol 2006; 53:721-7.
22. Protas AM, Bonna A, Kopera E, Bal W. Selective peptide bond hydrolysis of cysteine peptides in the presence of Ni(II) ions. J Inorg Biochem 2011; 105:10-6.
23. Kaminskaia NV, Kostic NM. New selectivity in peptide hydrolysis by metal complexes. platinum(II) complexes promote cleavage of peptides next to the tryptophan residue. Inorg Chem 2001; 40:2368-77.
24. Regan L. The Design of Metal-Binding Sites in Proteins. Annu Rev Biophys Biomol Struct 1993; 22:257-81.
25. Koch KA, Pena MM, Thiele DJ. Copper-binding motifs in catalysis, transport, detoxification and signaling. Chem Biol 1997; 4:549-60.
26. Katz AK, Shimoni-Livny L, Navon O, Navon N, Bock CW, Glusker JP. Copper-binding motifs: Structural and theoretical aspects. Helv Chim Acta 2003; 86:1320-38.
27. Hegg EL, Burstyn JN. Hydrolysis of Unactivated Peptide-Bonds by a Macrocyclic Copper(II) Complex - Cu([9]Anen(3))Cl-2 Hydrolyzes Both Dipeptides and Proteins. J Am Chem Soc 1995; 117:7015-6.
28. Chaderjian WB, Chin ET, Harris RJ, Etcheverry TM. Effect of copper sulfate on performance of a serum-free CHO cell culture process and the level of free thiol in the recombinant antibody expressed. Biotechnol Prog 2005; 21:550-3.
29. Salinas BA, Sathish HA, Shah AU, Carpenter JF, Randolph TW. Buffer-dependent fragmentation of a humanized full-length monoclonal antibody. J Pharm Sci 2010; 99:2962-74.
30. Smith MA, Easton M, Everett P, Lewis G, Payne M, Riveros-Moreno V, Allen G. Specific cleavage of immunoglobulin G by copper ions. Int J Pept Protein Res

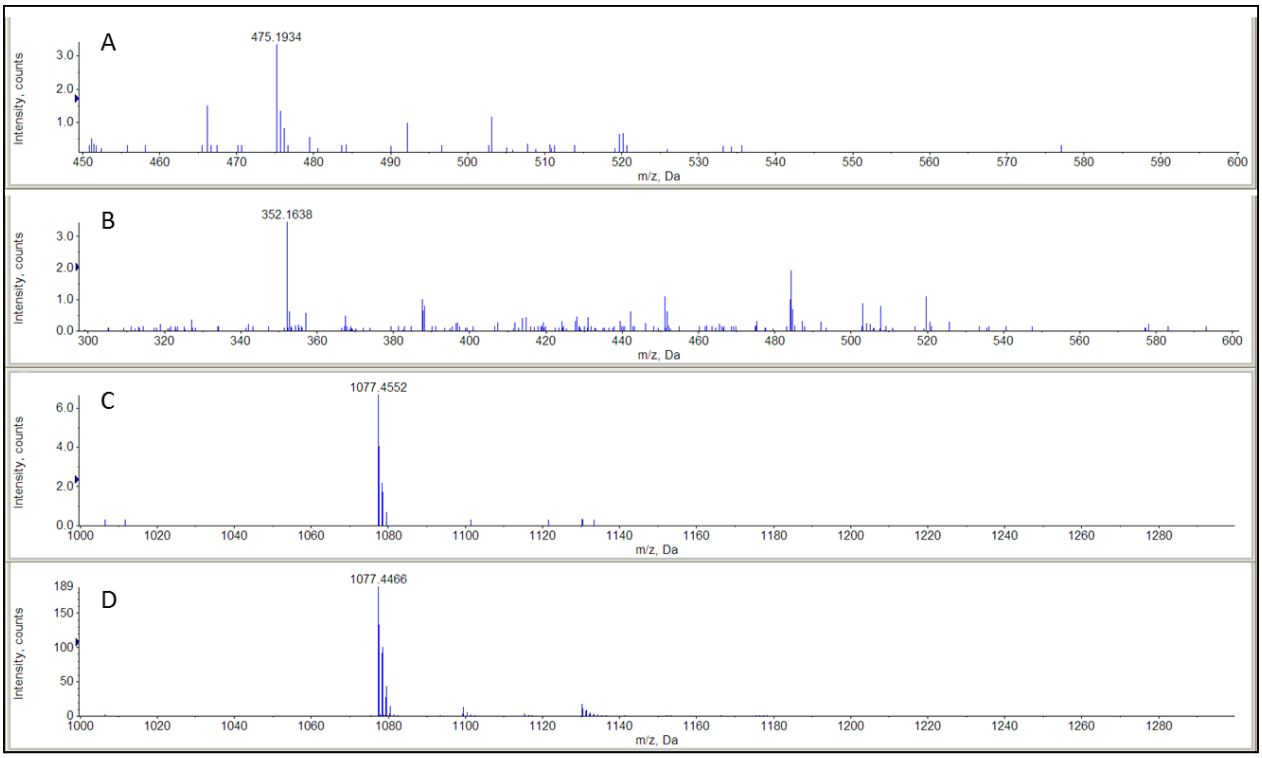
1996; 48:48-55.
31. Allen G, Campbell RO. Specific cleavage of histidine-containing peptides by copper(II). Int J Pept Protein Res 1996; 48:265-73.
32. Cunningham BC, Wells JA. High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis. Science 1989; 244:1081-5.
33. Kamerzell TJ, Li M, Arora S, Ji JA, Wang YJ. The relative rate of immunoglobulin gamma 1 fragmentation. J Pharm Sci 2011; 100:1341-9.
34. Giles NM, Watts AB, Giles GI, Fry FH, Littlechild JA, Jacob C. Metal and redox modulation of cysteine protein function. Chem Biol 2003; 10:677-93.
35. Huang X, Cuajungco MP, Atwood CS, Hartshorn MA, Tyndall JD, Hanson GR, Stokes KC, Leopold M, Multhaup G, Goldstein LE, et al. Cu(II) potentiation of alzheimer abeta neurotoxicity. Correlation with cell-free hydrogen peroxide production and metal reduction. J Biol Chem 1999; 274:37111-6.
36. Kamerzell TJ, Middaugh CR. Two-dimensional correlation spectroscopy reveals coupled immunoglobulin regions of differential flexibility that influence stability. Biochemistry 2007; 46:9762-73.

37. Rustandi RR, Wang Y. Use of CE-SDS gel for characterization of monoclonal antibody hinge region clipping due to copper and high pH stress. Electrophoresis 2011; 32:3078-84.
38. Ouellette D, Alessandri L, Piparia R, Aikhoje A, Chin A, Radziejewski C, Correia I. Elevated cleavage of human immunoglobulin gamma molecules containing a lambda light chain mediated by iron and histidine. Anal Biochem 2009; 389:107-17.
39. Schultz J, Allison H, Grice M. Specificity of the cleavage of proteins by dilute acid. I. Release of aspartic acid from insulin, ribonuclease, and glucagon. Biochemistry 1962; 1:694-8.
40. Oliyai C, Borchardt RT. Chemical pathways of peptide degradation. IV. Pathways, kinetics, and mechanism of degradation of an aspartyl residue in a model hexapeptide. Pharm Res 1993; 10:95-102.
41. Zhang L, Mei Y, Zhang Y, Li S, Sun X, Zhu L. Regioselective cleavage of myoglobin with copper(II) compounds at neutral pH. Inorg Chem 2003; 42:492-8.
42. Masuoka J, Saltman P. Zinc(II) and copper(II) binding to serum albumin. A comparative study of dog, bovine, and human albumin. J Biol Chem 1994; 269:25557-61.
43. Viles JH, Cohen FE, Prusiner SB, Goodin DB, Wright PE, Dyson HJ. Copper binding to the prion protein: structural implications of four identical cooperative binding sites. Proc Natl Acad Sci U S A 1999; 96:2042-7.
44. Tainer JA, Getzoff ED, Richardson JS, Richardson DC. Structure and mechanism of copper, zinc superoxide dismutase. Nature 1983; 306:284-7.
45. Sadler PJ, Tucker A, Viles JH. Involvement of a lysine residue in the N-terminal Ni2+ and Cu2+ binding site of serum albumins. Comparison with Co2+, Cd2+ and Al3+. Eur J Biochem 1994; 220:193-200.
46. Bogdanowich-Knipp SJ, Chakrabarti S, Williams TD, Dillman RK, Siahaan TJ. Solution stability of linear vs. cyclic RGD peptides. J Pept Res 1999; 53:530-41.
47. Khossravi M, Borchardt RT. Chemical pathways of peptide degradation: IX. Metal-catalyzed oxidation of histidine in model peptides. Pharm Res 1998; 15:1096-102.
48. Sreedhara A, Lau K, Li C, Hosken B, Macchi F, Zhan D, Shen A, Steinmann D, Schoneich C, Lentz Y. Role of surface exposed tryptophan as substrate generators for the antibody catalyzed water oxidation pathway. Mol Pharmaceutics 2013; 10:278-88.
49. Sankararamakrishnan R, Verma S, Kumar S. ATCUN-like metal-binding motifs in proteins: identification and characterization by crystal structure and sequence analysis. Proteins 2005; 58:211-21.
50. Saphire EO, Parren, P.W., Pantophlet, R., Zwick, M.B., Morris, G.M., Rudd, P.M., Dwek, R.A., Stanfield, R.L., Burton, D.R., Wilson, I.A. Crystal structure of neutralizing humam IGG against HIV-1: a template for vaccine design. 2001: 1155-9
51. The PyMOL Molecular Graphics System, Version 1.5.0.5 Schrodinger, LLC.

SUPPLEMENTAL FIGURES

S1. RP-HPLC duplicate injections for linear peptide SCDKTHTC at pH 6 (MES buffer).

Time point	% Parent peak		% Difference |Inj 1- Inj 2|
	Injection 1	Injection 2	
T0	99.33	98.87	0.46
9 hrs	64.24	66.01	1.77
24 hrs	40.24	41.85	1.61



S2. Linear peptide SCDKTHTC degradation products by LC-MS. 0.15 mM Cu^{2+} acetate buffer, pH 5.0. Spectra of A) 948 Da, B) 702 Da species, C) 1076 Da isomer, and D) parent peptide.

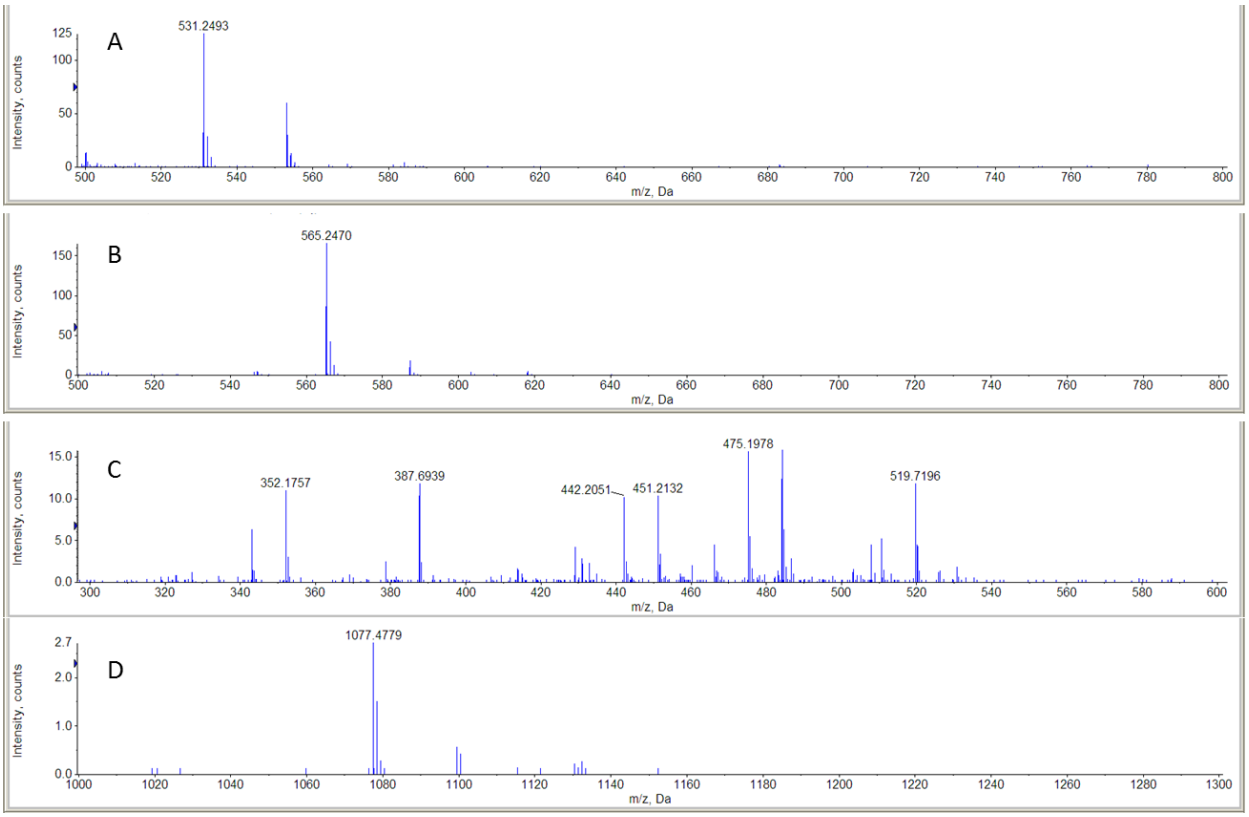


Figure S3. Linear peptide SCDKTHTC degradation products by LC-MS 0.15 mM Cu^{2+} HEPES buffer, pH 7.0. Spectra of A) 530 Da species, B) 564 Da species, C) 773 Da species, and D) 1076 Da isomer.

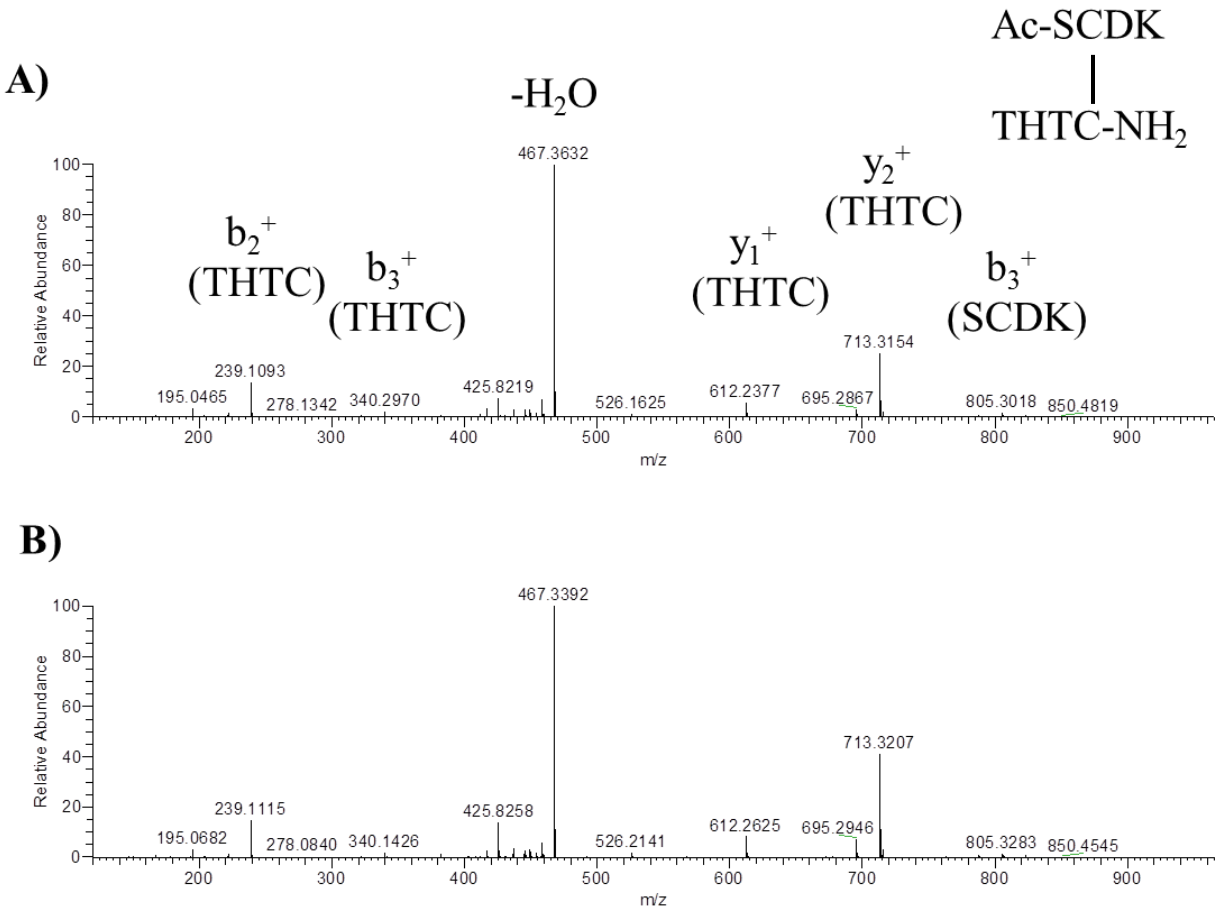


Figure S4. Cyclic peptide degradation products by LC-MS A) 0.15 mM Cu^{2+} acetate buffer, pH 5.3, 408 h (17 days), 50 °C. 0.15 mM Cu^{2+}, HEPES buffer, pH 7, 52 h, 50 °C contains similar results (data not shown). B) Cyclic peptide at time zero tryptic digest in water with no Cu^{2+}. Detection at 214 nm.

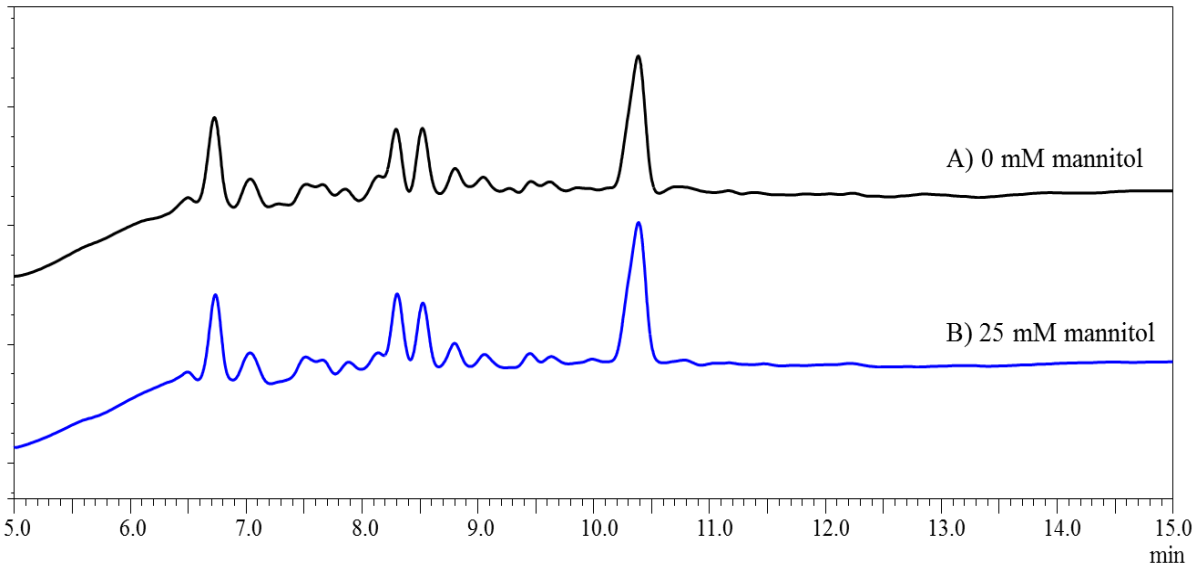


Figure S5. RP-HPLC chromatographic profile of linear peptide in 20 mM sodium acetate pH 5.3, 60 °C, after 50 h incubation with Cu^{2+} and A) no mannitol and B) 25 mM mannitol